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The Glucagon-sensitive Adenyl Cyclase System in Plasma Membranes of Rat Liver

IV. EFFECTS OF GUANYL NUCLEOTIDES ON BINDING OF ^{125}I-GLUCAGON

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SUMMARY

Studies have been made of the effects of nucleotides on binding of ^{125}I-glucagon at its specific binding sites in plasma membranes of rat liver.

GTP and GDP, equally and at a minimal concentration of 0.05 μM, stimulate the rate and degree of dissociation of bound labeled hormone, decrease uptake of glucagon by the membranes, and decrease the affinity of the binding sites for glucagon. These effects of the nucleotides are concentration-dependent, reversible, and rapid in onset. Divalent metal ions are not required for the actions of the nucleotides which act equally at 0° or 30°. 5′-Methylene guanylyl-diphosphonate, a nonphosphorylating analogue of GTP, mimicked the effects of GTP or GDP on glucagon binding although at 100 times the concentration of the natural nucleotides. Based on these observations and the finding that the nucleotides do not act competitively with glucagon, it is suggested that GTP or GDP regulate glucagon binding by an allosteric type of action. This action of the guanyl nucleotides is inhibited by a sulfhydryl reagent (*p*-chloromercuribenzoate), which also inhibits binding of glucagon. Sodium fluoride, which stimulates adenyl cyclase activity in liver membranes, has no effect on either the binding of glucagon or on the actions of guanyl nucleotides on this process.

ATP, ADP, UTP, and CTP act similarly to the guanyl nucleotides on the glucagon binding process but only at concentrations greater than 0.1 mM. Cyclic 3′,5′-GMP, 5′-GMP, and the corresponding adenine nucleotides are inactive on the glucagon-binding process.

In the previous study (1) correlations were found between binding of ^{125}I-glucagon to specific binding sites in rat liver plasma membranes and activations of adenyl cyclase by the hormone, suggesting that the specific binding sites are components of the adenyl cyclase system. It was also found that EDTA stimulated dissociation of bound labeled glucagon. This phenomenon plus the marked temperature dependence of the binding process suggested that the reaction between hormone and binding site is complex and may be influenced by other factors.

During the course of investigating whether binding of glucagon may be affected by ATP, the substrate for adenyl cyclase, and by other components of the medium used for assaying adenyl cyclase activity, it was discovered that guanyl nucleotides, at extraordinarily low concentrations, altered the binding of ^{125}I-glucagon. These findings are detailed in the present report.

EXPERIMENTAL PROCEDURES

Only those materials and methods not described in the preceding papers (1–3) are documented in the present communication.

Materials—ATP, ADP, AMP, and GMP were obtained from Sigma; GTP, GDP, UTP, and CTP from P-L Laboratories; cyclic 3′,5′-AMP from Calbiochem; cyclic 3′,5′-GMP from Schwarz BioResearch. 5′-Guanylyl methylene diphosphonate was purchased from Miles Laboratory; *p*-chloromercuribenzoate from Sigma. ^{8}GTP-α-^{32}P was obtained from International and Nuclear Corporation.

Measurement of Hydrolysis of GTP by Nucleotidases in Plasma Membranes—Plasma membranes (25 μg of protein) were incubated at 30° in medium containing 2.5% bovine serum albumin (Fraction V), 20 mM Tris-HCl, pH 7.6, and 50 μM GTP-γ-^{32}P (specific activity, 60,000 cpm per nmole) in a final volume of 0.125 ml. After 2 min of incubation, the reaction was stopped by the addition of 1 ml of 0.16 M perchloric acid containing 2 μmoles of P_i. The mixture was allowed to stand for 10 min at 0° and was then centrifuged at 2000 rpm in an International refrigerated centrifuge. $^{32}P_i$ liberated during incubation was isolated by the following modification of the method of Sugino and Miyoshi (4). To 0.5 ml of the supernatant fluid was added 0.75 ml of a solution containing 8 mM triethylamine-HCl (prepared by titrating a 0.2 M solution with 1 N HCl to pH 5.0), 6.4 mM ammonium molybdate, and 16 mM perchloric acid. After standing in the cold for 10 min the precipitate of triethylamine-phosphomolybdate (4) was collected on 0.45 μ Millipore filters and washed with 1 ml of ice-cold water. The filters were allowed to dry in counting vials and were dissolved along with the precipitate in 1 ml of acetone. $^{32}P_i$ was determined in 15 ml of Bray's

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TABLE I

Effects of ATP, Mg^{++}, and ATP-regenerating system (creatine kinase-phosphocreatine) on uptake of glucagon by plasma membranes

Incubations were carried out at 30° for 20 min. The standard medium for incubation contained, in 0.125 ml, 25 μg of membrane protein, 1 mM EDTA, 2.5% albumin, and 20 mM Tris-HCl, pH 7.6. The complete medium contained, in addition, 5 mM $MgCl_2$, 3.5 mM ATP, 20 mM phosphocreatine, and 1 mg of creatine phosphokinase (Sigma, 44 units per mg) per ml. Both media contained 4.2 nM ^{125}I-glucagon (10^6 cpm per pmole). Separation of bound from free labeled glucagon was carried out as described elsewhere (1).

Incubation medium	Glucagon bound
	pmoles/mg protein
Standard	1.05
Complete	0.50
−ATP-regenerating system	0.58
$-MgCl_2$	0.52
−ATP	1.13

scintillation fluid (5) in a liquid scintillation counter. Based on the specific activity of labeled GTP, the quantity of radioactivity was converted to picomoles of P_i liberated. Activities of the nucleotidase (or nucleotidases) are expressed as nanomoles of P_i liberated per min per mg of membrane protein. Protein was determined by the method of Lowry *et al.* (6), as modified in the previous study (1), using crystalline bovine albumin as standard.

RESULTS

Effects of ATP on Binding of Labeled Glucagon to Liver Plasma Membranes—Addition of 3.2 mM ATP, 5 mM $MgCl_2$, and an ATP-regenerating system (creatine kinase and phosphocreatine) to the standard incubation medium (2.5% albumin, 1 mM EDTA, 20 mM Tris-HCl, pH 7.6) used in the previous binding studies (1) forms the complete medium used for assaying adenyl cyclase in liver plasma membranes (2). Binding of ^{125}I-glucagon to the membranes was reduced by 50% in the complete medium compared to the standard medium, as shown in Table I. This reduction was caused by ATP since its omission from the complete medium resulted in restoration of binding to levels observed in the standard medium.

Effects of Nucleotides on Uptake or Dissociation of Bound Labeled Hormone from Liver Membranes—Under adenyl cyclase incubation conditions, UTP, CTP, and GTP, at 3 mM, decreased uptake of glucagon by liver membranes and stimulated dissociation of bound labeled glucagon (Table II). It is seen that GTP was the most effective nucleotide, causing complete dissociation of bound hormone within 15 min of incubation. In subsequent experiments it was found that ATP and the pyrimidine nucleotides did not affect either uptake or dissociation of glucagon from the membranes at concentrations less than 0.1 mM. The effects of GTP and ATP, at varying concentrations, on the dissociation process are described in Fig. 1. GTP stimulated dissociation of glucagon at a concentration as low as 0.05 μM whereas the minimal effective concentration of ATP was between 100 μM and 500 μM. It will also be noted that 0.05 mM GTP stimulated complete release of bound labeled glucagon.

Also tested were the effects of various known metabolites of GTP on the dissociation process (Table III). At the same concentration, 1.0 μM, GDP was as effective as GTP in stimulating

TABLE II

Effects of various triphosphonucleotides on uptake of labeled glucagon and dissociation of bound labeled glucagon from liver membranes

Plasma membranes (75 μg of protein) were incubated in 0.375 ml of complete medium (described in legend to Table I) containing ^{125}I-glucagon (4.5 nM) with the indicated nucleotides either omitted or added at final concentration of 3.0 mM. In studies of uptake of glucagon, incubations were at 30° for 15 min. In studies of dissociation of bound labeled glucagon, incubations were carried out as above but with no added nucleotides during the first 15 min of incubation. At 15 min, duplicate 50-μl aliquots were withdrawn for assay of bound glucagon, and 125-μl aliquots were added to 10 μl of a solution containing unlabeled glucagon and the indicated nucleotides to give final concentrations of 5.0 μM and 3.0 mM, respectively. Incubations were continued for 15 min at 30°, after which 50-μl aliquots were again taken for assay of bound labeled glucagon. Bound labeled glucagon was assayed by Method A described in the previous report (1). Calculations of percentage of labeled glucagon dissociated were made as described in legend to Fig. 1. Results are the average of two experiments ± S.E.M.

Additions	Uptake of glucagon	Bound ^{125}I-glucagon dissociated
	pmoles/mg protein	%
None	1.15 ± 0.03	49 ± 2
CTP	0.75 ± 0.04	65 ± 3
UTP	0.71 ± 0.04	75 ± 4
ATP	0.59 ± 0.02	75 ± 4
GTP	0.43 ± 0.02	100 ± 1

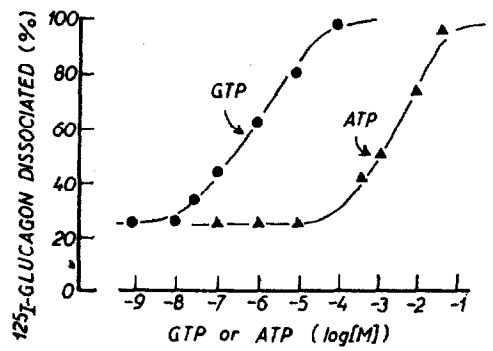

FIG. 1. Effects of varying concentrations of GTP and ATP on dissociation of ^{125}I-glucagon from plasma membranes. Incubations were carried out in two stages. In the first stage, plasma membranes (150 μg of protein) were incubated at 30° in a final volume of 0.750 ml of incubation medium containing 2.5% albumin, 1 mM EDTA, 4 nM ^{125}I-glucagon (10^6 cpm per pmole), and 20 mM Tris-HCl, pH 7.6. After 15 min, duplicate 50-μl aliquots were taken for measuring bound labeled glucagon as described previously (1). In the second stage of incubation, GTP or ATP were added simultaneously with unlabeled glucagon (final concentration, 5.0 μM) to give the indicated concentrations. After 15-min incubation at 30°, 50-μl aliquots were taken for measuring bound labeled glucagon as above. The percentage of bound labeled glucagon dissociated during the second stage was calculated from the difference between the amount of bound labeled glucagon in the first and second stages of incubation as described previously (1).

the dissociation of bound labeled glucagon; other experiments showed these nucleotides to be equally effective at 0.05 μM. Cyclic 3′,5′-GMP and 5′-GMP did not alter dissociation of bound glucagon or uptake of the labeled hormone by liver mem-

branes. In similar experiments it was found that ATP and ADP, at 3 mM, exerted equivalent effects on dissociation of bound labeled glucagon; cyclic 3′,5′-AMP and 5′-AMP, at 3 mM, did not alter binding of labeled glucagon.

Effects of GTP on Time Course of Dissociation of Bound Glucagon—The stimulatory effects of GTP, at concentrations ranging from 0.1 μM to 0.1 mM, on dissociation of bound labeled glucagon were rapid in onset; significant stimulation was observed within 1 min of the addition of GTP to the incubation medium (Fig. 2). Although initial rates were too rapid to measure accurately, it can be seen that both the rates and degree of dissociation were dependent upon the initial concentration of GTP.

The effects of GTP on dissociation of bound labeled glucagon were not dependent upon the presence of unlabeled glucagon in the incubation medium. As shown in Fig. 3, omission of unlabeled glucagon (5.0 μM) reduced only the extent of dissociation in 3 min of incubation; the difference from that observed in the presence of unlabeled glucagon probably reflects reassociation of labeled glucagon at its binding sites.

Effects of Various Concentrations of GTP on Levels of Bound Glucagon—As was noted previously for the effects of ATP (Table I), GTP also decreased the levels of glucagon taken up by liver membranes. In Fig. 4, it is seen that the effects of low concentrations of GTP on uptake of labeled glucagon were less marked than on dissociation of bound hormone. However, the concentration of GTP required for minimal effects on uptake and dissociation were essentially the same, about 0.05 μM.

Effects of GTP on Affinity of Binding Sites for Glucagon—GTP (1.0 μM) did not modify the number of binding sites for labeled glucagon but shifted the concentration of glucagon required for half-maximal binding from 2.5 nM to about 6.0 nM (Fig. 5). Similar studies were not carried out at higher concentrations of GTP, although it is evident from the marked effects of 1.0 mM GTP on the uptake of glucagon (Fig. 4) that the apparent affinity of the binding sites for glucagon is decreased further at this concentration of the nucleotide.

Effects of GTP on Time Course of Binding in Presence and Absence of EDTA—Studies of the effects of GTP on binding were carried out in the presence of EDTA (1.0 mM) in all of the studies reported thus far. As shown in Fig. 6, 1 μM GTP, in the presence or absence of EDTA, decreased the initial uptake of glucagon. However, in the absence of EDTA, the inhibitory effects of GTP on uptake of glucagon were not sustained. After 10 min of incubation, uptake of glucagon returned to levels approaching that attained in the absence of GTP; in the presence of EDTA the effects of 1 μM GTP were sustained for at least 25 min. It will be noted that GTP decreased the time at which a constant level of bound glucagon is attained (5 min) compared to the control (about 15 min).

EDTA did not alter the time course of uptake of glucagon or the amount bound.

Effects of EDTA on Hydrolysis of GTP by Nucleotidase in Plasma Membranes—Plasma membranes of rat liver have been shown to contain magnesium or calcium ion-dependent nucleo-

TABLE III

Effects of GTP, GDP, GMP, and cyclic 3′,5′-GMP on dissociation of bound ^{125}I-glucagon from liver membranes

Plasma membranes (75 μg of protein) were incubated in 0.375 ml of medium containing 2.5% albumin, 1 mM EDTA, 4.5 nM ^{125}I-glucagon (10^6 cpm per pmole), and 20 mM Tris-HCl, pH 7.6. Incubations were for 15 min at 30°. Duplicate 50-μl aliquots were withdrawn for assay of bound glucagon (Method A in previous study (1)), followed by addition of 10 μl of a solution containing unlabeled glucagon and the indicated nucleotides to give final concentrations of 5 μM and 1 μM, respectively. Incubations were continued for 15 min at 30°, after which 50-μl aliquots were again assayed for bound labeled glucagon.

Additions	Bound ^{125}I-glucagon dissociated
	%
None	37
5′-GMP	35
Cyclic 3′,5′-GMP	36
GDP	65
GTP	63

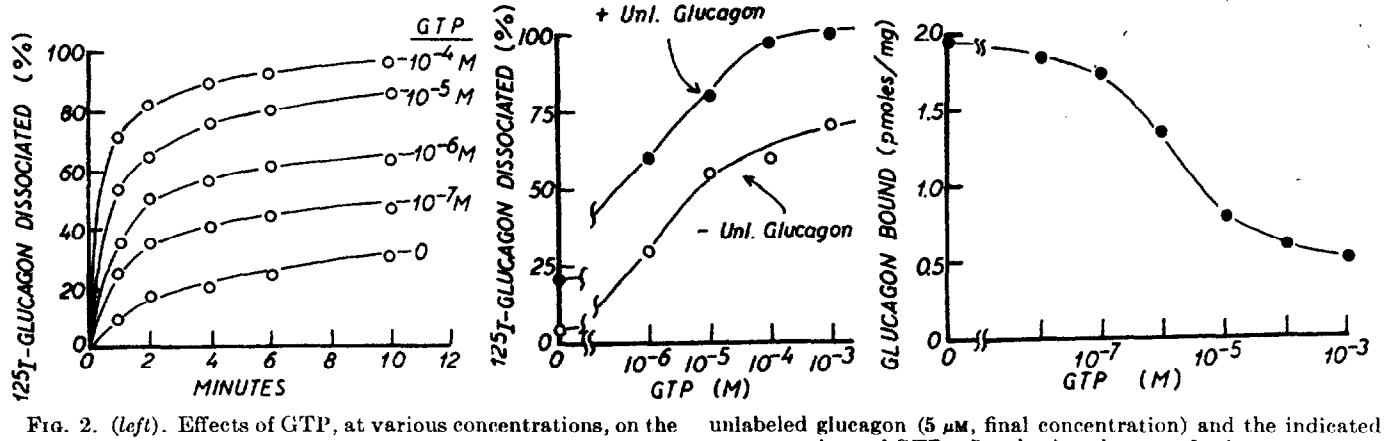

FIG. 2. (*left*). Effects of GTP, at various concentrations, on the time course of dissociation of ^{125}I-glucagon from plasma membranes. Experiments were carried out in the same manner and under the incubation conditions described in legend to Fig. 1.

FIG. 3 (*center*). Effects of GTP on dissociation of bound ^{125}I-glucagon from plasma membranes in the presence and absence of unlabeled glucagon. Experiments were conducted in the same manner and under the identical incubation conditions described for the first stage of incubation in Fig. 1. In the second stage, incubations were continued at 30° in the absence and presence of unlabeled glucagon (5 μM, final concentration) and the indicated concentrations of GTP. Incubation time was 3 min.

FIG. 4 (*right*). Effects of various concentrations of GTP on uptake of ^{125}I-glucagon. Plasma membranes (25 μg of protein) were incubated in 0.125 ml of medium containing 2.5% albumin, 5.2 nM ^{125}I-glucagon (0.4×10^6 cpm per pmole), Tris-HCl, pH 7.6, and the indicated concentrations of GTP. Incubation time was 15 min at 30°. The quantity of glucagon bound to plasma membranes was determined from the amount of radioactivity bound and the specific activity of the labeled glucagon, as described previously in detail (1).

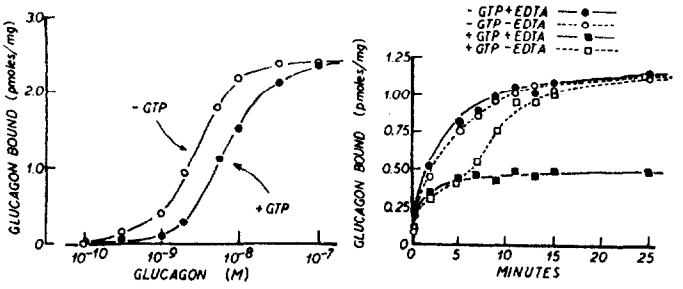

FIG. 5 (*left*). Effects of GTP on the uptake of varying concentrations of ^{125}I-glucagon by plasma membranes. Plasma membranes (25 μg of protein) were incubated for 20 min at 30° in 0.125 ml of medium containing 2.5% albumin, 1 mM EDTA, 20 mM Tris-HCl, pH 7.6, and the indicated concentrations of ^{125}I-glucagon (320,000 cpm per pmole). GTP, when present, was 4 μM. The method of separating bound from free labeled hormone and calculations of amount of glucagon bound per mg of membrane protein are described in Reference 1.

FIG. 6 (*right*). Effects of GTP on the time course of binding of glucagon in the presence and absence of EDTA. Plasma membranes (25 μg of protein) were incubated at 30° in 0.125 ml of medium containing 2.5% albumin, 5.4 nM ^{125}I-glucagon (320,000 cpm per pmole), and Tris-HCl, pH 7.6. When present, the concentrations of EDTA and GTP were 1 mM and 1 μM, respectively. The method of separating bound from free labeled hormone and calculations of amount of glucagon bound per mg of protein are described in Reference 1.

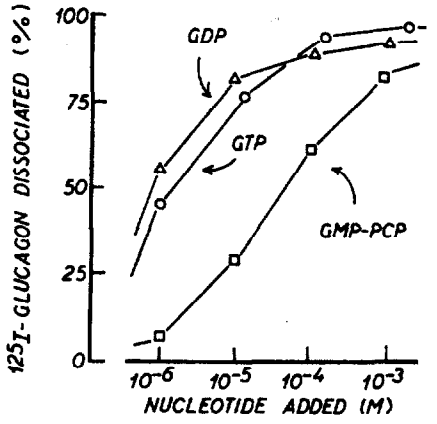

FIG. 7. Effects of various concentrations of GTP, GDP, and GMP-PCP on dissociation of bound labeled glucagon from plasma membranes. Experiments were carried out in the absence of EDTA but otherwise under the same manner and under the incubation conditions described in legend to Fig. 1 with the exception that either GTP, GDP, or GMP-PCP were added simultaneously with unlabeled glucagon (5 μM) during the second stage of incubation.

tidases (7). Since EDTA had a permissive effect on the action of low concentrations of GTP, it was suspected that the chelator may prevent the breakdown of GTP by removing metal ions bound to the membranes. We examined this possibility by measuring the hydrolysis of GTP-γ-^{32}P, 20 μM, in the same medium used for the binding studies and with the same concentration of plasma membranes (200 μg of protein per ml). In the absence of EDTA, GTP was hydrolyzed at a rate of 14 nmoles per min per mg of protein. EDTA, at 1 mM, inhibited the hydrolysis of GTP by 96%, suggesting that its permissive effect on the actions of GTP may be due to inhibition of nucleotidases present in liver membranes. In studies that will be reported later in detail, it has been found that 5 mM Mg^{++} or Ca^{++} doubled the rate of hydrolysis of GTP.

TABLE IV

Effects of GTP on dissociation of bound labeled glucagon from liver membranes incubated at 0° and 30°

Liver membranes (0.2 mg per ml) were incubated in medium containing 2.5% albumin, 20 mM Tris-HCl, pH 7.6, and 4.5 nM ^{125}I-glucagon for 15 min at 30°. Half of the suspension was placed in an ice bath and incubated at 0° for 15 min in presence of 5 μM unlabeled glucagon with or without GTP (1.0 μM). The other half was incubated with the same additions but at 30°. The percentage of bound labeled glucagon dissociated during the second stage of incubation was determined as described previously (1).

Incubation temperature	Dissociation of bound labeled glucagon	
	−GTP	+GTP
	%	
0°	4	46
30	9	49

TABLE V

Effects of CMB on uptake and dissociation of labeled glucagon in liver membranes and on effects of GTP on these processes

Plasma membranes (0.2 mg per ml) were incubated in medium containing 2.5% albumin, 1 mM EDTA, 20 mM Tris-HCl, pH 7.6, 4.5 nM ^{125}I-glucagon. When present, CMB and GTP were 0.2 mM and 1.0 μM, respectively, and were added at zero time in uptake studies and at the moment of addition of unlabeled glucagon in studies of dissociation. Incubations were for 15 min at 30° during both types of studies which were carried out as described in the legend to Table II.

CMB	Uptake of glucagon		Bound ^{125}I-glucagon dissociated	
	−GTP	+GTP	−GTP	+GTP
	pmoles/mg protein		%	
−	1.18	0.68	38	75
+	0.46	0.34	35	35

Comparative Effects of 5'-Guanylyl Methylene Diphosphonate, GTP, and GDP on Dissociation of Bound Glucagon—The effects of GMP-PCP[1] (an analogue of GTP containing a carbon atom in place of oxygen between the β and γ phosphate groups) were compared with those of GDP and GTP on dissociation of bound glucagon. GMP-PCP can only be hydrolyzed to give 5'-GMP (8), which is inactive on glucagon binding. As shown in Fig. 7, GMP-PCP also stimulated dissociation of bound glucagon although at concentrations 100 times that of either GTP or GDP which had essentially equivalent actions at all concentrations.

Other Characteristics of GTP Actions on Binding of Glucagon—The effects of GTP on dissociation of bound labeled glucagon were equivalent at either 0° or 30° of incubation as illustrated in Table IV. In the absence of GTP, dissociation was slight at either temperature (less at 0°, see also Reference 1). It should be noted that EDTA, which stimulates dissociation of bound labeled glucagon (1), was omitted in these experiments.

Shown in Table V are the effects of the sulfhydryl reagent, CMB, at 0.2 mM, on both uptake of glucagon and dissociation of bound labeled glucagon, and on the actions of GTP on these processes. CMB inhibited binding of glucagon by 61% and either abolished or inhibited markedly the effects of GTP on

[1] The abbreviations used are: GMP-PCP, 5'-guanylyl methylene diphosphonate; CMB, *p*-chloromercuribenzoate.

either uptake of glucagon or dissociation of bound labeled hormone.

In studies not recorded, it was found that sodium fluoride (10 mM), in the presence or absence of Mg^{++} (5 mM) or of EDTA (1 mM), did not alter either the processes of binding (uptake or dissociation of bound labeled hormone) or the actions of GTP on these processes.

DISCUSSION

The previous studies (1, 3) showed that agents such as phospholipase A, urea, and detergents diminished both the binding of glucagon and activation of adenyl cyclase by the hormone. While these correlations help to establish the relationship between binding sites and the adenyl cyclase system and provide some insight into the chemical and physical properties of the binding sites, the destructive actions of these agents on both processes do not permit the conclusion that the binding process is necessarily or uniquely involved in the activation of adenyl cyclase by glucagon. For this aspect of the problem, more meaningful correlations can be derived from studies with agents that stimulate or alter both processes in a nondestructive manner. Two phenomena observed in this series of studies seem to fit this criterion. One is the stimulatory effect of EDTA on both activation of adenyl cyclase by glucagon (2) and dissociation of bound glucagon (1). The other, reported in this study, is the action of GTP or GDP, at concentrations approaching that at which glucagon binds or activates adenyl cyclase in liver membranes, on the binding sites for glucagon. The following study will show that guanyl nucleotides stimulate the response of adenyl cyclase to glucagon (9). The mechanism by which EDTA or the guanyl nucleotides alter binding or activation of adenyl cyclase remains unknown, but it can be suggested, in the case of EDTA, that membrane-bound metal ions may be involved in both processes. The actions of the guanyl nucleotides appear to differ both qualitatively and quantitatively from that of EDTA on the binding process.

GTP and GDP, equally and at concentrations as low as 0.05 μM, stimulated both the rate and degree of dissociation of bound glucagon, decreased the uptake of submaximal concentrations of glucagon, and decreased the apparent affinity of the binding sites for glucagon. The relatively slow method of measuring binding in these studies did not permit assessment of the effects of the nucleotides on the initial rates of association or dissociation of the hormone at its binding sites. It can be stated, however, that the nucleotides induce a change in the state or properties of the binding sites leading to a more rapid attainment of constant, although lower levels of bound glucagon and to a change in the apparent affinity of the binding sites for glucagon.

Although the mechanism by which the guanyl nucleotides alter the state of the glucagon binding sites remains unknown, the following observations suggest that they act through binding and not by phosphorylation of the material that binds glucagon. Both GTP and GDP were equally effective at the same concentrations, indicating that interconversion of one nucleotide to the other is not required for their effects. There was no requirement for a divalent ion as might be expected for a phosphorylating mechanism. Indeed, EDTA sustained the effects of GTP on binding by inhibiting the breakdown of the nucleotide by Mg^{++}- or Ca^{++}-requiring nucleotidases present in the membranes. GMP-PCP, a nonphosphorylating analogue of GTP, mimicked the actions of GTP or GDP, although at concentrations 100 times that of the natural compounds. The lower apparent affinity of the analogue for the glucagon binding sites may be related to the recent report (10) that the bond angles of the diphosphonate bond (P—C—P) are significantly different from those of the pyrophosphate bond (P—O—P). It is also possible that the analogue exerts its action by inhibiting the breakdown of endogenous, membrane-bound guanyl nucleotides. Finally, GTP stimulated dissociation of bound labeled glucagon equally at 0° and 30°, suggesting that the actions of GTP are related to a binding rather than an enzymatic process. In this regard, the finding that cyclic 3′,5′-GMP and 5′-GMP did not alter binding of glucagon rules out the possibility that nucleotidases or guanyl cyclase, the latter reported to be present in particulate fractions of rat liver (11), may be responsible for the actions of GTP or GDP.

At low concentrations, GDP and GTP did not show competitive interaction with glucagon at the binding sites. This was evident from the finding that the nucleotides act at 0.05 μM even in the presence of 5 μM glucagon in the incubation medium. It would appear, therefore, that the nucleotides exert an allosteric type of action on the glucagon binding sites. The effects of GTP were reversible and concentration dependent, suggesting that breakdown by nucleotidases or other factors that influence metabolism or binding of guanyl nucleotides may regulate the actions of the nucleotides on the glucagon-binding process. Both binding of glucagon and the actions of GTP on binding were inhibited by a sulfhydryl reagent, which raises the possibility that regulation of both processes could be affected through modification of sulfhydryl groups.

It was also found in this study that ATP, ADP, and the pyrimidine nucleotides, UTP and CTP, also affected glucagon binding in the same manner as GTP or GDP although at concentrations at least four orders of magnitude higher than the minimal effective concentration of the guanyl nucleotides. It is possible that the effects of the other nucleotides are due to contaminated guanyl nucleotides. In any case, the consequence of the effects of the adenine nucleotides on glucagon binding is that studies of the specific effects of guanyl nucleotides on the response of adenyl cyclase to glucagon necessitates the use of low concentrations (less than 0.5 mM) of ATP, the substrate for adenyl cyclase.

The following paper (9) examines the question of whether the effects of the guanyl nucleotides on the binding of glucagon bear relationship to the actions of glucagon on the adenyl cyclase system in rat liver plasma membranes.

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